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Role of the VLA-4 integrin in leucocyte recruitment and bronchial hyperresponsiveness in the guinea-pig

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Abstract

Guinea-pigs sensitized to ovalbumin develop airway eosinophilia and bronchial hyperresponsiveness to acetylcholine 24 h following ovalbumin challenge. We have used an antibody to the very late antigen-4 (VLA-4) integrin (HP 1/2) to investigate the role of this integrin in the recruitment of leucocytes to the airways and to study the link between eosinophilia and the development of bronchial hyperresponsiveness. In this model, HP 1/2 reduced the number of eosinophils recovered from bronchoalveolar lavage fluid, as well as reducing the activity of the enzyme eosinophil peroxidase in both bronchoalveolar lavage fluid and whole lung extracts. However, HP 1/2 did not reduce bronchial hyperresponsiveness. The results suggest that after antigen challenge, the VLA-4 integrin is involved in the recruitment of leucocytes to the airways at the level of the vascular endothelium, but increased numbers of eosinophils in lavage fluid or in the lung tissue are not an essential correlate of bronchial hyperresponsiveness in this model.

Keywords: Bronchial hyperresponsiveness; Eosinophilia; Adhesion molecule; VLA-4 integrin

1. Introduction

Leucocyte migration to inflammatory sites is an essential factor in the pathogenesis of chronic inflammatory diseases. Inflammation in the asthmatic lung is characterized by an infiltrate of leucocytes, particularly eosinophils in the airways (Dunnill, 1960). Adhesion of leucocytes to the vascular endothelium is a crucial event in the migratory process, and involves specialized adhesion molecules (Springer, 1990). These include the integrin family of proteins, expressed on leucocytes, and their ligands, members of the immunoglobulin supergene family, expressed on the vascular endothelium. The integrin, very late antigen-4 (VLA-4), is expressed on eosinophils and other leucocytes, but not on neutrophils (Walsh et al., 1991; Weller et al., 1991; Dobrina et al., 1991), hence, adhesion of VLA-4 to its endothelial counterpart, vascular cell adhesion molecule-1 (VCAM-1), may provide a selective pathway for the migration of eosinophils. Recently Weg et al. (1993) reported that an antibody to VLA-4 inhibited eosinophil accumulation in guinea-pig skin. In addition, although there is no ligand for VLA-4 on airway epithelial cells (Bloemen et al., 1993), the same antibody inhibited allergen-induced recruitment of eosinophils to the airways in guinea-pigs (Pretolani et al., 1994), suggesting that the VLA-4 integrin has a role in the recruitment of eosinophils to the guinea-pig airways.

In asthmatic patients, increased eosinophil numbers in the lung have been reported to correlate with bronchial hyperresponsiveness (Wardlaw et al., 1988), and it has been suggested that bronchial hyperresponsiveness results from airway epithelial damage caused by the secreted products of eosinophils which have migrated into the airways (Barnes, 1986). However, there is a growing body of evidence indicating that these two phenomena can occur independently of one another (Chapman et al., 1993).

It has been demonstrated that ovalbumin sensitization and challenge of guinea-pigs results in the accumulation of leucocytes, mainly eosinophils, in bronchoalveolar lavage fluid and lung tissue (Dunn et al., 1988) and in the development of bronchial hyperresponsiveness to acetylcholine, 24 h after challenge (Milne and

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Piper, 1994a). Previous work using this model has shown that bronchial hyperresponsiveness can occur independently of eosinophilia in lung tissue or bronchoalveolar lavage fluid (Milne and Piper, 1994a,1995). In the present study, using this experimental model of asthma, we have investigated the effect of antibody to the VLA-4 integrin in order to confirm the importance of this adhesion pathway in the recruitment of eosinophils to the lung and bronchoalveolar lavage fluid. In addition, the effect of the antibody to VLA-4 on the development of bronchial hyperresponsiveness was examined, in order to establish whether bronchial hyperresponsiveness and eosinophilia are causally linked.

2. Materials and methods

2.1. Sensitization to ovalbumin

Male Duncan-Hartley guinea-pigs (180–200 g) were sensitized to ovalbumin over a 2-week period, starting on day 0 with an injection of 1 mg ovalbumin and 1 mg aluminium hydroxide in 1 ml saline (i.p.) and *Bordetella pertussis* vaccine (0.25 ml i.p.). On day 7, the animals were given a booster injection of 0.1 mg ovalbumin and 0.1 mg aluminium hydroxide in 0.1 ml saline (i.p.). On day 14 the guinea-pigs were challenged with an aerosol of ovalbumin (0.1% w/v in saline) for 1 h under protection of pyrilamine (10 mg/kg i.p.).

2.2. Assessment of airway function

On day 15, 24 h following ovalbumin challenge, the animals were anaesthetized (midazolam: 7.5 mg/kg s.c. and a mixture of fentanyl: 0.63 mg/kg and fluanisone: 20 mg/kg i.m.). Animals were ventilated with room air (10 ml/kg, 1 Hz) via a tracheal cannula connected to a respiratory pump (Palmer Miniature Ideal). Air flow was monitored by a pneumotachograph (type 0000, Fleisch, Switzerland) in line with the respiratory pump. Pressure changes within the thorax were measured via an intrathoracic cannula and the differential pressure between the trachea and the thorax was measured with a differential pressure transducer (PM45-24, Validyne, USA). Airway resistance and dynamic compliance were calculated from pressure and flow measurements with a computerized pulmonary monitoring system (Mumed, London, UK). The jugular vein was cannulated to allow administration of acetylcholine and arterial blood pressure was monitored via a cannula inserted into the carotid artery.

2.3. Bronchoalveolar lavage

Following measurement of airway function, the animals were killed with an overdose of pentobarbitone (i.v.), and the lungs were lavaged with 40 ml (8 × 5 ml) of cold (4°C) buffer (PBS: 10 mM phosphate buffer containing 2.7 mM potassium chloride, 137 mM sodium chloride and 0.25% (w/v) bovine serum albumin pH = 7.4). This procedure gave an average recovery of 86% of instilled fluid. Samples of bronchoalveolar lavage fluid (200 μl) were stored at −20°C (1–2 months) for measurement of eosinophil peroxidase levels by colorimetric assay (see below).

2.4. Cell counting

Bronchoalveolar lavage fluid was centrifuged at 400 × *g* for 10 min at 4°C. The resulting pellet was resuspended in 2 ml of ammonium chloride (0.87% w/v in distilled water) and left at room temperature for 10 min to allow lysis of red blood cells. 20 ml of PBS was added and the suspension was centrifuged. The pellet was resuspended in 1 ml of PBS and the number of leucocytes was determined by counting in a Neubauer chamber. Differential cell counts were performed on fixed slides of the cell suspension, which were stained with Diff-Quik (Baxter Healthcare). The relative proportions of leucocyte subtypes were determined by counting 400–500 cells/slide.

2.5. Colorimetric assay of eosinophil peroxidase in bronchoalveolar lavage fluid

Samples of bronchoalveolar lavage fluid (uncentrifuged) were thawed on ice, diluted 1:2.5 in Tris buffer (0.05 M, pH = 8) and sonicated over ice for 1 min. Eosinophil peroxidase levels in sonicate were assayed based on a method used by Strath et al. (1985). Briefly, 100 μl of the sonicate was incubated with an equal volume of substrate (*o*-phenylenediamine dihydrochloride 1.0 mM in Tris buffer with 0.1% Triton X-100 and 0.35 M H_2O_2) for 30 min at 20°C in microtitre plates. The reaction was stopped by addition of 50 μl of 4 M H_2SO_4. The absorbance was read on a spectrophotometer at 492 nm and the amount of eosinophil peroxidase activity present in each sample was estimated from standard curves constructed using known amounts of the enzyme horseradish peroxidase (6.25–250 ng/ml; Sigma, Poole, Dorset, UK). The results of the eosinophil peroxidase assay are expressed as μg equivalents of horseradish peroxidase per ml of bronchoalveolar lavage fluid.

2.6. Colorimetric assay of eosinophil peroxidase in whole lung homogenates

In a separate set of experiments, the eosinophil peroxidase content of lung was measured to assess the number of eosinophils in the lung tissue. This method was based on that of Goldblum et al. (1985). Guinea-

pigs were killed by overdose of pentobarbitone (i.p.). 10 ml of heparinized saline (4°C) was injected into the right ventricle to flush the lungs of blood. The lungs were removed and all subsequent procedures carried out at 4°C. Lungs were chopped into small cubes in Tris buffer, chopped lungs blotted on filter paper, 2 g weighed into a test tube, and 10 ml of Tris buffer added. The samples were then homogenized (Polytron) for 10 s followed by sonication for 1 min. Lung samples were centrifuged at $40000 \times g$ for 5 min, and the supernatant decanted and centrifuged at $48000 \times g$ for 60 min. The resulting pellet was resuspended in 3 ml of Tris buffer. This was centrifuged at $12000 \times g$ for 5 min and the supernatant, which was diluted 1:5, was assayed as described above. Protein content was measured using the method of Lowry et al. (1951). The results were expressed as μg peroxidase equivalents per mg lung protein.

2.7. Treatment with anti-VLA-4 antibody

HP 1/2 (Biogen, Cambridge, MA, USA) is a purified mouse anti-human VLA-4 antibody (immunoglobulin G_1) which has previously been shown to cross-react in the guinea-pig (Weg et al., 1993), a finding which was confirmed in our laboratory (results not shown).

Sensitized guinea-pigs were given intravenous doses of HP 1/2, 1 h prior to ovalbumin challenge, and again 4 h after challenge. Two dose levels of the antibody were used, 3 mg/kg and 10 mg/kg. Assays for eosinophil peroxidase in bronchoalveolar lavage fluid and lung tissue were performed only in animals treated with the lower dose of 3 mg/kg HP 1/2. A control antibody was also tested at the same doses, this was the purified mouse myeloma protein, MOPC21 (immunoglobulin G_1, Celltech, Slough, UK).

2.8. Experimental design and analysis

Experiments were performed with groups of 10 guinea-pigs, 5 animals receiving test antibody and 5 receiving control antibody. Comparisons of mean values from each group were made between treatments using a Student's unpaired t-test and a P value of < 0.05 was taken to indicate significant differences between means.

3. Results

3.1. Effect of antibody treatment on bronchial hyperresponsiveness to acetylcholine

Sensitization alone did not affect basal resistance (sensitized, 113 ± 3; unsensitized, 118 ± 3 cm $H_2O/l/s$) or increases in airway resistance to acetylcholine, which were not different to those in unsensitized animals (data not shown). At 24 h post-ovalbumin challenge of sensitized guinea-pigs, basal resistance was unchanged (127 ± 9 cm $H_2O/l/s$) but there was a bronchial hyperresponsiveness to acetylcholine (Fig. 1a).

Treatment of guinea-pigs with either 3 mg/kg or 10 mg/kg of HP 1/2 or the control antibody, MOPC21, had no effect on the bronchial hyperresponsiveness to acetylcholine (Fig. 1b and c). The effect of antibody treatment on dynamic elastance was similar to that on resistance measurements and is not shown.

Table 1
Cellular composition of bronchoalveolar lavage fluid in unsensitized or sensitized guinea-pigs, unchallenged or 24 h post-challenge, treated with HP 1/2 or MOPC21

	Leucocyte numbers ($\times 10^6$) in bronchoalveolar lavage fluid				
	Total	Macrophages	Lymphocytes	Neutrophils	Eosinophils
Unsensitized	9.2 ± 2.3	8.3 ± 2.3	0.4 ± 0.1	0.01 ± 0.01	0.5 ± 0.1
Sensitized	13.5 ± 1.7	11.0 ± 1.3	1.0 ± 0.3	0.07 ± 0.07	1.5 ± 0.4 *
24 h	30.9 ± 3.1 **	16.1 ± 1.2 *	2.2 ± 0.4 **	1.9 ± 0.9	10.9 ± 2.3 **
3 mg/kg:					
MOPC	43.4 ± 6.8	22.8 ± 3.3	1.5 ± 0.3	1.4 ± 1.2	17.8 ± 2.9
HP 1/2	30.4 ± 4.4	20.5 ± 2.0	1.8 ± 0.4	1.1 ± 1.0	7.8 ± 1.4 +
10 mg/kg:					
MOPC	40.7 ± 5.6	25.2 ± 4.0	1.3 ± 0.4	0.5 ± 0.3	13.8 ± 1.5
HP 1/2	22.1 ± 2.6 +	16.9 ± 1.6	0.8 ± 0.1	0.8 ± 0.2	3.6 ± 0.9 ++

The values in the Table are the mean ($\pm$S.E.M.) cell counts from 5 animals in each condition. The total cell number increased 24 h after challenge, the greatest increase was in eosinophils, about 20-fold. Treatment with 3 mg/kg of HP 1/2 caused a reduction in the eosinophils at this time point, but there was no change in the total cell number. At the higher dose of 10 mg/kg of HP 1/2, there was a further reduction in the eosinophil numbers, and a reduction in the total cell numbers compared to MOPC21 treatment. Comparisons were made using Student's t-test; * $P < 0.05$, ** $P < 0.01$, mean values significantly different from unsensitized values and + $P < 0.05$, ++ $P < 0.01$, mean values significantly different from values following MOPC21 treatment.

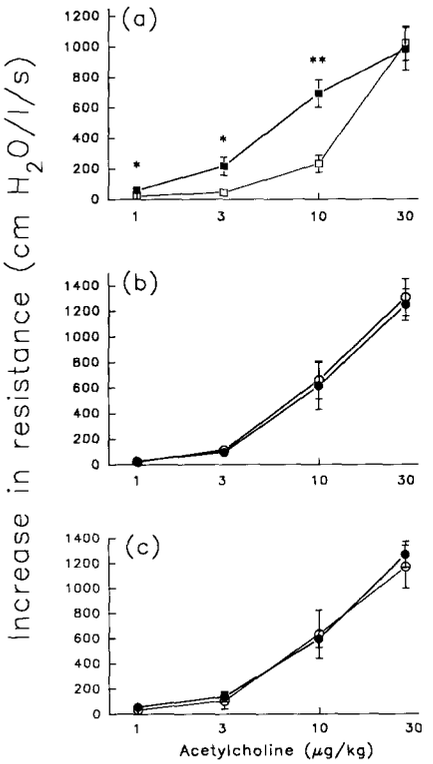

Fig. 1. Effect of ovalbumin sensitization and challenge (a) (■) on airway responsiveness to acetylcholine versus unsensitized (□), (b) following treatment with 3 mg/kg HP 1/2 (●) or MOPC21 (○) and (c) following treatment with 10 mg/kg HP 1/2 (●) or MOPC21 (○). Mean ± S.E.M. are shown, except where the error is smaller than the size of the symbol. 24 h post-ovalbumin challenge of sensitized guinea-pigs, there is a bronchial hyperresponsiveness to acetylcholine that is not affected by treatment with 3 or 10 mg/kg HP 1/2. Sensitization alone has no effect on the response to acetylcholine (data not shown). * $P < 0.05$, ** $P < 0.01$, for sensitized challenged versus unsensitized guinea-pigs.

3.2. Effect of antibody treatment on leucocyte accumulation in bronchoalveolar lavage fluid

At 24 h post-challenge, the number of eosinophils in bronchoalveolar lavage fluid had increased by 20-fold, lymphocytes by 5-fold and macrophages by 2-fold over unsensitized animals, while there was no change in the neutrophil population (Table 1). Treatment with 3 mg/kg of HP 1/2 caused a reduction in the eosinophil numbers of about 56% although there was no change in the total number of cells compared to MOPC21 treated animals. At the higher dose of 10 mg/kg of HP 1/2 there was a further reduction in the eosinophil population of about 74% compared to MOPC21 treatment, and in addition, there was a 46% reduction in the total number of cells in bronchoalveolar lavage fluid (Table 1). Treatment with the control antibody MOPC21 at both dose levels tended to increase the total number of cells and the eosinophil population when compared to numbers in animals not receiving antibody treatment, but this was not statistically significant.

3.3. Effect of antibody treatment on eosinophil peroxidase in bronchoalveolar lavage fluid and lung homogenates

The levels of eosinophil peroxidase in bronchoalveolar lavage fluid of sensitized guinea-pigs 24 h post-challenge were greater than those in untreated animals (Fig. 2a). A similar increase in eosinophil peroxidase activity post-challenge was seen in lung homogenates with mean values about 4-fold greater than those in the lungs taken from untreated guinea-pigs (Fig. 2b).

Treatment with HP 1/2 (3 mg/kg) decreased eosinophil peroxidase levels in bronchoalveolar lavage fluid relative to those after MOPC21 (Fig. 2a). In lung homogenates, treatment with 3 mg/kg HP 1/2 reduced the eosinophil peroxidase activity almost to those levels seen in unsensitized animals (Fig. 2b).

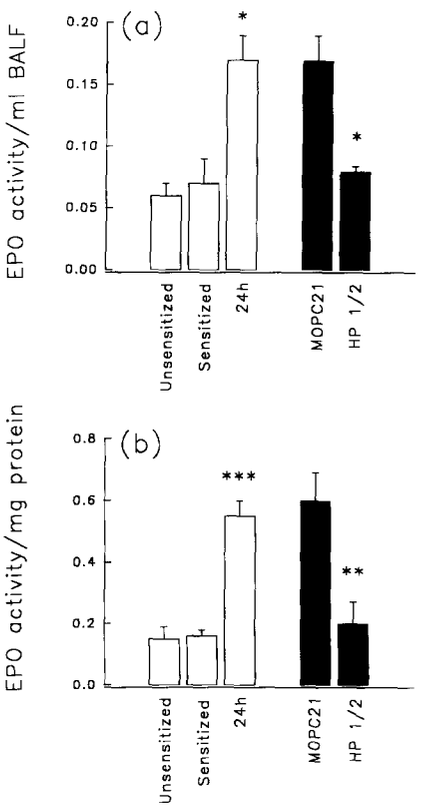

Fig. 2. Eosinophil peroxidase activity in bronchoalveolar lavage fluid (a) or whole lung extracts (b) from untreated, sensitized or sensitized guinea-pigs 24 h after challenge (unfilled bars) treated with 3 mg/kg of either MOPC21 or HP 1/2 (filled bars). Sensitization and challenge leads to an increase in the eosinophil peroxidase activity in both bronchoalveolar lavage fluid and whole lung extracts (8). Treatment with HP 1/2 reduced the eosinophil peroxidase activity in bronchoalveolar lavage fluid and in whole lung.

4. Discussion

Sensitization of guinea-pigs to ovalbumin and subsequent challenge induced a bronchial hyperresponsiveness to acetylcholine and an accumulation of eosinophils in bronchoalveolar lavage fluid and lung tissue measured at 24 h following challenge (Milne and Piper, 1994a,b), and with this model we have here, through the use of an antibody to VLA-4, assessed the involvement of the VLA-4 integrin in the events described.

The major effect of the antibody was to decrease strikingly the eosinophil accumulation in either bronchoalveolar lavage fluid or in lung tissue. This effect was unlikely to have been due to a reduction in circulating eosinophils, since Weg et al. (1993) demonstrated that treatment of guinea-pigs with HP 1/2 (1, 3 or 10 mg/kg) did not alter the percentage of circulating radiolabelled eosinophils. Indeed, intravenous administration of HP 1/2 to sensitized challenged guinea-pigs (Pretolani et al., 1994) and mice (Nakajima et al., 1994) increased significantly the number of blood eosinophils. In bronchoalveolar lavage fluid, the halving of eosinophil accumulation with no effect on other types of leucocyte demonstrated a selective effect on eosinophils compatible with reports using other systems in this species (Weg et al., 1993; Pretolani et al., 1994). Nakajima et al. (1994) showed a reduction in eosinophils accumulating in mouse trachea with a similar reduction in lymphocyte numbers. In the present study, a higher amount of antibody, although inducing a further fall in eosinophil numbers, tended to affect other cell types as well. At least two explanations are possible; either this antibody at the higher level exhibits enough non-selective binding to interact with integrins on other leucocyte types or the greater reduction in eosinophilia itself decreases the chemotactic factors attracting the other cell types present. Nevertheless eosinophils were, at both levels of antibody, the most susceptible to the effects of the antibody to VLA-4. A similarly selective inhibition of eosinophil accumulation in bronchoalveolar lavage fluid was achieved in this model with antibodies to the CD18 family of integrins (Milne and Piper, 1994a). It is therefore clear that interference with either the VLA-4 or the CD18 integrins markedly decreased the numbers of eosinophils in bronchoalveolar lavage fluid after challenge. In contrast, antibody to the VLA-4 integrin also decreased eosinophils in lung tissue, as assessed by the eosinophil peroxidase activity, whereas those to the CD18 integrins did not affect eosinophil peroxidase activity in the lung which remained high after challenge. This discrepancy could reflect the different distribution of the ligands for the leucocyte integrins: VCAM-1, the ligand for VLA-4, is found on endothelium but not epithelium, whereas one of the ligands for the CD18 integrins, ICAM-1, is known to be located on airway epithelial cells. In order to appear in bronchoalveolar lavage fluid in the lumen of the airway, leucocytes have to cross the vascular endothelium, migrate across the lung tissue and finally cross the epithelial barrier. In the present study, the decrease in eosinophil number in bronchoalveolar lavage fluid was accompanied by a decrease in eosinophils in lung tissue, suggesting that eosinophils were not able to cross the initial barrier, i.e., the vascular endothelium, in the presence of the antibody to VLA-4. This is in agreement with the view that the VLA-4 integrin is important for the migration of eosinophils at the level of the vascular endothelium, by binding to VCAM-1, the expression of which is cytokine inducible (Elices et al., 1990). However, interference with the CD18 integrins (through the antibodies R15.7 and 6.5E) allowed the accumulation of eosinophils in lung tissue but prevented their subsequent appearance in bronchoalveolar lavage fluid (Milne and Piper, 1994a) implying a critical role for CD18 integrins at the final barier, at the airway epithelium. Our results would therefore support the postulate of selective recruitment of eosinophils via the VLA-4 integrin as this pathway, in our model, was crucial for the entry and accumulation of eosinophils in lung tissue.

Apart from VCAM-1, fibronectin is also a ligand for VLA-4 but at a separate site on the integrin molecule (Elices et al., 1990). The antibody used in the present study is known to interact with this binding site on the integrin as well as the VCAM-1 site (Weg et al., 1993). It has been reported that eosinophil binding to fibronectin results in prolonged survival (Anwar et al., 1993). However, this interaction would take place in the interstitial tissue, and, since in our studies there were very few eosinophils penetrating the endothelium following treatment with the antibody, the contribution of this additional effect would not be obvious.

Recently, Moser et al. (1992) demonstrated in vitro the importance of priming of eosinophils by cytokines in their migration via VLA-4/VCAM-1. In their study, penetration of endothelial cell layers stimulated by interleukin-4 was dependent on priming of eosinophils from normal donors by granulocyte/macrophage-colony stimulating factor, interleukin-3 or interleukin-5, and eosinophils from patients with allergic asthma and atopic dermatitis showed an increased capacity to penetrate these cell layers. We have previously shown interleukin-5 to be of importance in our model of airway inflammation in the guinea-pig, since an antibody to interleukin-5 inhibits both eosinophilia in bronchoalveolar lavage fluid and the development of bronchial hyperresponsiveness (Milne and Piper, 1994b).

Models of airway inflammation in other species may have different cytokine profiles generated on antigen

challenge which may, in part, account for differences in the effect of antibody to VLA-4 on cell accumulation observed between species. For example, in sheep, HP 1/2 did not consistently affect the composition of leucocytes in bronchoalveolar lavage fluid (Abraham et al., 1994) and similarly in the rat, Rabb et al. (1994) demonstrated that there was no reduction in cell numbers in bronchoalveolar lavage fluid. This suggests that, in these cases, there is a lesser role for the VLA-4 integrin in the recruitment of leucocytes to the airways, indicating the involvement of alternative pathways. The earlier timepoint (8 h) examined in the study by Rabb et al. (1994) may also explain the dissimilar effects of anti-VLA-4 antibody on cell recruitment.

In contrast to the marked effects on eosinophils, the antibody to VLA-4 had no effect on the concomittant bronchial hyperresponsiveness in our model even at the higher dose where a less selective and more widespread inhibition of leucocyte numbers was achieved. A similar result was obtained with one antibody to the CD18 integrin, 6.5E, which also reduced eosinophils in bronchoalveolar lavage fluid by 80% without affecting bronchial hyperresponsiveness (Milne and Piper, 1994a). At that time it was possible to postulate that hyperresponsiveness was less dependent on eosinophils in bronchoalveolar lavage fluid and more related to eosinophils in lung tissue since treatment with 6.5E was accompanied by unchanged, high eosinophil peroxidase activity in lung. However, in the present conditions, the antibody HP 1/2 prevented accumulation of eosinophils in both bronchoalveolar lavage fluid and lung and consequently bronchial hyperresponsiveness was not causally related to eosinophils in bronchoalveolar lavage fluid or in lung. The hyperresponsiveness and eosinophilia in this model would thus appear to be two independent though coincidental phenomena, perhaps with a common, cytokine, cause.

Bronchial hyperresponsiveness in response to allergen was inhibited by treatment with antibody to VLA-4 in sheep (Abraham et al., 1994) despite a lack of consistent effect on cell recruitment to the lung. Although in the present study there was a lack of effect of antibody on bronchial hyperresponsiveness, the results of Abraham et al. (1994) are in agreement with those presented here in that bronchial hyperresponsiveness and eosinophilia do not appear to occur in parallel.

In contrast, Pretolani et al. (1994) demonstrated that antibody to VLA-4 markedly inhibited the increased bronchopulmonary responses to methacholine as well as airway eosinophilia in bronchoalveolar lavage fluid and bronchial tissue in the guinea-pig in response to antigen. While the inhibitory effects on eosinophil recruitment and eosinophil peroxidase in bronchoalveolar lavage fluid are in agreement with our findings, the effect on bronchial hyperresponsiveness is at odds. One explanation for this discrepancy may be in the different methods of sensitization used. Pretolani et al. (1994) administered the antigen (ovalbumin) by aerosol and in the absence of adjuvants. This resulted in a much smaller response to antigen challenge, with a 2-fold increase in the number of eosinophils recruited into bronchoalveolar lavage fluid and a 2-fold shift in agonist reactivity. In the model described here, a 20-fold increase in eosinophils in bronchoalveolar lavage fluid and a 5-fold shift in agonist reactivity was observed. It would appear that the use of the adjuvants aluminium hydroxide and *Bordetella pertussis* vaccine contributed to the more pronounced inflammatory response described here and it is possible that these methodological considerations may have led to the different results observed with antibody treatment.

In conclusion, our results support a role for the VLA-4 integrin in the selective recruitment of eosinophils to the inflamed lung, although it is clear that this process is also dependent upon the release of cytokines which can prime eosinophils and induce the expression of adhesion molecules on endothelium. In addition, in this model we have dissociated the presence of eosinophils in bronchoalveolar lavage fluid and lung tissue from the development of bronchial hyperresponsiveness.

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